Beyond Matching Dim Expression Densities with Bright Fluorochromes: A Novel Practical Approach to Design Multicolor Applications

Michael Kapinsky, Beckman Coulter Life Science, Inc., Krefeld, Germany

INTRODUCTION

Multiparametric cytometry utilizing 8 and more colors has become a common research tool. An ever growing menu of available fluorochromes and laser configurations seems to disclose boundless possibilities but also raised the bar of operational experience required to obtain valid results and satisfying detection limits in multicolor panels. Several papers offer rough rules for fluorochrome selection and highlight pitfalls and caveats [1, 2]. However, systematic approaches to manage the demands of panel design within complex crosstalk patterns framed by a greatly varying brightness across the dye portfolio are scarce if not absent.

The scope of the novel concept presented herein is to overcome trial-and-error strategies and to provide instead a systematic and easy-to-use approach to maximize sensitivity in multicolor applications by smart panel design also facilitating accurate data analysis. Derived from physical facts regarding fluorochrome properties, photon counting statistics, digital compensation and hardware performance as well as through consideration of biological features such as antigen expression characteristics and antigen patterns a set of 6 easily applicable rules was developed. Furthermore, several terms are introduced such as “silent dyes”, “untouchable channels”, “distortion factors” and “crosstalk indices”.

MATERIALS & METHODS

To develop and demonstrate the set of 6 rules step-by-step single and multiple antibody stainings were conducted in human whole blood. All antibody conjugates were obtained from Beckman Coulter. Samples were processed according to a stain-lyse-wash procedure using VersaLyse and IOTest Fixative Solution (40:1) and acquired on a Gallios* Flow cytometer equipped with 3 Lasers capable to detect 12 parameters at 20-bit resolution (>10^6 physical channels). The flow cytometer was set up according to the manufacturers recommendations. Obtained data was analyzed using Kaluza* Software Version 1.2.

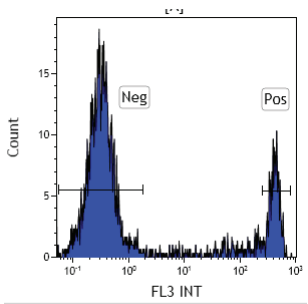
RESULTS

The Distortion Map

The brightness of fluorochromes on a given hardware is a determinant of sensitivity and can be assessed with conjugates of common specificities such as CD4 or CD8. Mean fluorescence intensities of negative and positive populations on the primary detector (signal-to-noise ratio, staining index; Figure 1) are normalized according to:

Signal-to-Noise Ratio (SNR):
MFI(pos) / MFI(neg),
420.15/0.37 = 1136

Staining Index (SI):
(MFI(pos)-MFI(neg))/2*SD(neg),
(420.15-0.37)/2*0.23 = 913



Gate	X-AMean	X-Stdev
All	78,37	161,02
Neg	0,37	0,23
Pos	420,15	82,32

Figure 1: Measures of dye brightness using an ECD-conjugate of CD8 (clone B9.11, gate on lymphocytes in lysed whole blood)

crosstalk of dyes into secondary channels results in spreading of the compensated negative population in the secondary channel which - in line with discrete event counting statistics – results from the higher absolute statistical spread found for an increased number of photons (background + crosstalk > background) . The linear relation between $\log_{10}$ decades of lost sensitivity in a secondary channel and mean intensity of a crosstalking signal is represented by the hinged quadrant axis and can be referred to as “distortion factor” (Figure 2).

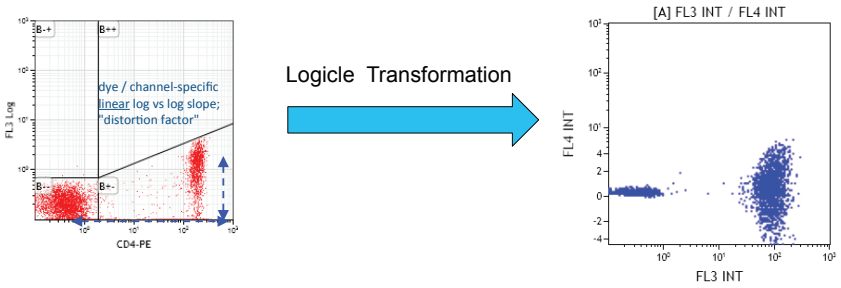


Figure 2: Crosstalk results in a loss of sensitivity in secondary channels

The resulting loss of sensitivity depends on the absolute amount of crosstalk but also on the bandpass used. The following paragraphs will try to further describe these influences and corresponding determinants.

Despite of their direct dependence on PMT voltages usually compensation factors are presented in order to characterize a dye’s crosstalk to secondary detectors. In contrast, an accurate scaling of absolute crosstalk amounts is provided by the “crosstalk index ” that is insensitive towards changes of PMT settings (linearity provided, Figure 2):

$\log_{10}$(SNR(MFI(secondary channel))) / $\log_{10}$(SNR(MFI(primary channel)))

(SN = signal-to-noise ratio; MFI = arithmetic mean intensity of fluorescence)

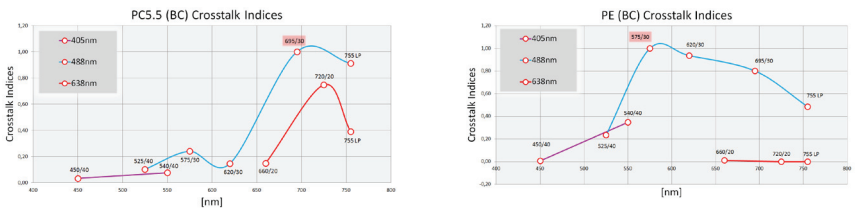


Figure 3: Typical PE (BC) and PC5.5 (BC) crosstalk indices from a Gallios with 3 Lasers / 12 parameters and standard filter set*

Crosstalk indices are vastly dye-specific and determine the spreading of a population compensated in a secondary channel for the dye’s crosstalk, respectively. However, there is a wavelength- or bandpass-dependency of absolute precision of photon detection that will also affect the spreading of compensated populations, i.e. with increasing wavelength of the photons captured by a PMT we find an increased spreading of compensated populations in secondary channels. Combining crosstalk indices and experimental data for λ-dependent distortion the following distortion map is obtained for a Gallios* cytometer with a standard filter set (Figure 4) and commonly used dyes. The elements of the grid indicate an estimated linear slope of $\log_{10}$ decades of lost sensitivity in a secondary channel per $\log_{10}$ decades of crosstalking signal in its primary channel. Dyes (columns) that only bear factors smaller than 0.10 may be referred to as “silent” dyes while detector-bandpass combinations (rows) solely with factors smaller than 0.10 may be referred to as “untouchable”. Factors from 0.15 up may exert considerable effects already at mid crosstalking signal intensity.

detectors / bandpass	silent	silent	Fluorochrome								
	PacBlue	KrO	FITC	PE	ECD	PC5.5	PC7	APC	APCA700	APCA750	
450/40	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	untouchable
540/40	0,08	0,00	0,06	0,06	0,01	0,00	0,00	0,00	0,00	0,00	untouchable
525/40	0,00	0,01	0,00	0,03	0,01	0,00	0,01	0,00	0,00	0,00	untouchable
575/30	0,00	0,01	0,12	0,00	0,09	0,05	0,06	0,00	0,00	0,00	
620/30	0,00	0,02	0,12	0,23	0,00	0,05	0,06	0,00	0,00	0,00	
695/30	0,00	0,01	0,08	0,21	0,26	0,00	0,08	0,05	0,10	0,01	
755LP	0,00	0,02	0,03	0,13	0,20	0,25	0,00	0,03	0,10	0,14	
660/20	0,00	0,00	0,00	0,00	0,03	0,09	0,01	0,00	0,14	0,16	
720/20	0,00	0,00	0,00	0,00	0,01	0,24	0,09	0,25	0,00	0,20	
755LP	0,00	0,00	0,00	0,00	0,00	0,12	0,15	0,12	0,21	0,00	

Figure 4: Distortion map for typical dyes on an Gallios with 3 Lasers / 12 parameters and standard filter set The grid elements indicate an estimated linear slope of $\log_{10}$ decades of lost sensitivity in a secondary channel per $\log_{10}$ decades of crosstalking signal in its primary channel.*

Literature Cited:
[1] Maecker HT, Frey T, Nomura LE, Trotter J. Selecting fluorochrome conjugates for maximum sensitivity. Cytometry A. 2004 Dec;62(2):169-73.
[2] Roederer M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. Cytometry 2001;45:194–205.

Co-expression patterns

Distortion through crosstalk and subsequent loss of sensitivity is often referred to as being only relevant when looking at co-expressed antigens while not being applicable to patterns with excluding antigens. These categories need to be refined as they do not drill down to all details and situations relevant to high sensitivity panel design (Figure 6): 1) excluding antigens, crosstalk has no impact on sensitivity; 2) modulated antigen co-expression, crosstalk causes loss of sensitivity and impedes discrimination of positive vs. negative events, FMO controls necessary; 3) discrete antigen co-expression, provided a high staining index for the co-expressed antigen a considerable amount of crosstalk may be tolerated; 4) parent and descendent, crosstalk from descendant to parent has no impact on sensitivity while the adverse case belongs either to 2) or 3); 5) multiple parents and descendants; resembles 4) but in contrast the descendent antigen is found in more than one parent population; crosstalk has no impact as long as the parents belong to different lineages or grandparent markers that could be gated within the antibody combination.

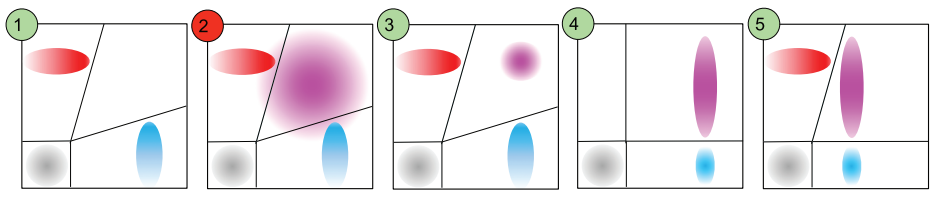


Figure 6: Expression patterns in a gated population of interest that determine achievable sensitivity within a given antibody conjugate combination

Furthermore the complete set of co-expressed markers beyond the direct parent antigen within a given panel frequently is overlooked although distortion may also be mediated via grandparent or non-parent markers.

6 complementary rules for high sensitivity multicolor panel design

Combining the distortion map and the crosstalk schemes of typical expression patterns the following set of rules can be framed. The additional “Crosstalk Complexity” rule aims at avoiding “ungateable” detection limits which may occur in case of several heterogeneously expressed markers crosstalking to a single secondary channel with a co-expressed antigen.

- **Old School**
  Weakly expressed antigen works best with bright dye, strongly expressed antigen works with all dyes
- **Channels**
  Weakly expressed antigen works best on “untouched“ channel, strongly expressed antigen works best with “silent“ dye
- **Exclusion**
  Allow crosstalk between excluding antigens (1)
- **Co-Expression**
  Avoid crosstalk between co-expressed antigens (2) (allow when coexpression is discrete and strong) (3)
- **Parent-Descendant**
  Allow crosstalk from descendant antigens to parent antigens, (4) (5) avoid vice-versa (2)
- **Crosstalk Complexity**
  Minimize complexity of crosstalk patterns

Proof-of-principle

Two panels with identical sets of specificities either being widely in line (left panel) or partially contradicting (right panel) the set of rules above were tested for their capability to detect delicate populations (Figure 7a and 7b).

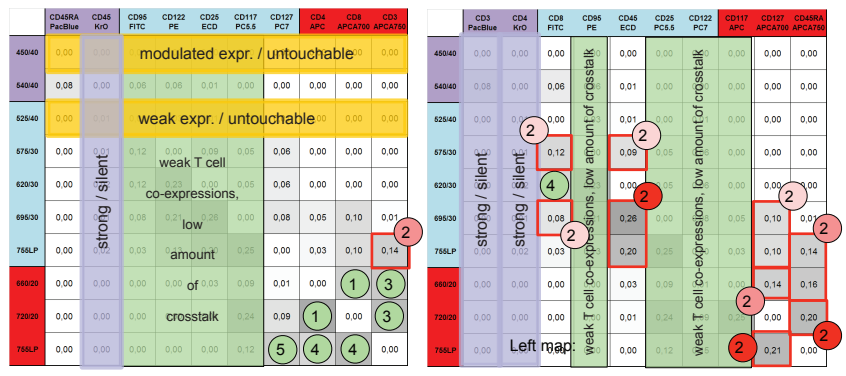


Figure 7a: Characterization of two conjugate combos with respect to panel design rules compliance and applicable expression patterns, respectively

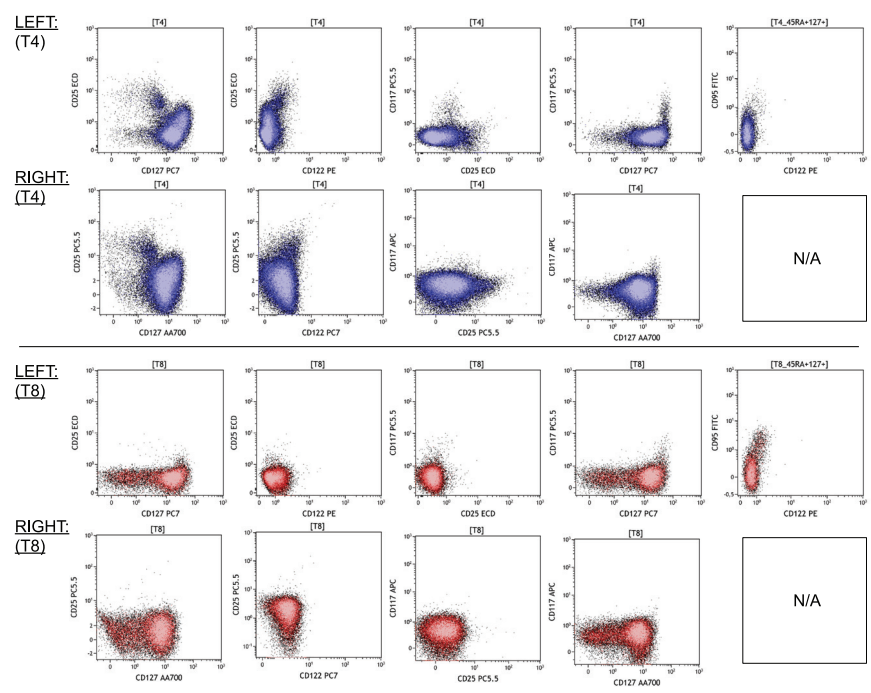


Figure 7b: The right panel strictly followed the rule “Old School”. However, the left panel focussed much more on compliance with the coexpression rules thus providing a better capability to detect delicate T cell subpopulations.

SUMMARY

The presented concept based on a distortion map - generated from dye crosstalk indices and experimental data on wavelength-dependent precision drift of PMT detectors – and 6 rules related to expression patterns, crosstalk patterns and fluorochrome brightness facilitates designing complex multicolor panels through a systematic approach. “Old School” guidelines that solely advise to match fluorochrome brightness with antigen density are insufficient and in many situations even counterproductive. Fluorochrome brightness is still relevant but only as one aspect among several other criteria and beyond just matching antigen density.